Role of Tissue Repair in Toxicologic Interactions among Hepatotoxic Organics

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It is widely recognized that exposure to combinations or mixtures of chemicals may result in highly exaggerated toxicity even though individual chemicals might not be toxic at low doses. Chemical mixtures may also cause additive or less than additive toxicity. From the perspective of public health, highly exaggerated toxicity is of significant concern. Assessment of risk from exposure to chemical mixtures requires knowledge of the underlying mechanisms. Previous studies from this laboratory have shown that nontoxic doses of chlordecone (10 ppm, 15 days) and carbon tetrachloride (CCl_4) (100 µl/kg) interact at the biologic interface, resulting in potentiated liver injury and 67-fold amplification of CCl_4 lethality. In contrast, although interaction between phenobarbital and CCl_4 leads to even higher injury, animal survival is unaffected because of highly stimulated compensatory tissue repair. A wide variety of additional experimental evidence confirms the central role of stimulated tissue repair as a decisive determinant of the final outcome of liver injury inflicted by hepatotoxicants. These findings led us to propose a two-stage model of toxicity. In this model, tissue injury is inflicted in stage one by the well-described mechanisms of toxicity, whereas in stage two the ultimate toxic outcome is determined by whether timely and sufficient tissue repair response accompanies this injury. In an attempt to validate this model, dose–response relationships for injury and tissue repair as opposing responses have been developed for model hepatotoxicants. Results of these studies suggest that tissue repair increases in a dose-dependent manner, restraining injury up to a threshold dose, whereupon it is inhibited, allowing an unrestrained progression of injury. These findings indicate that tissue repair is a quantifiable response to toxic injury and that inclusion of this response in risk assessment may help in fine-tuning prediction of toxicity outcomes. — *Environ Health Perspect* 106(Suppl 6):1307–1317 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1307-1317soni/abstract.html*

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People are concurrently or subsequently exposed to a range of toxic substances, and public concern regarding the adverse health effects of exposure to mixtures of chemicals has increased. These realities have heightened the need for exposure assessment, hazard identification, and risk characterization of chemical mixtures. Generally, safety evaluation of exposure to chemicals is based on studies of single individual chemicals. The large number of chemicals and their permutations and combinations dictates that mechanistic studies be conducted in a carefully designed research program to develop strategies to protect public health. From a perspective of public health, a major toxicologic issue is the possibility of unusual toxicity due to interaction of two or more toxic chemicals at individually harmless levels with environmental or occupational exposures.

In classic chemically induced toxicity studies only toxic injury has been measured as the end point of the mechanisms that inflict injury. In addition to toxic response, however, tissue repair, a simultaneous biologic compensatory response that accompanies chemical-induced injury, also needs due consideration (*1,2*). Several studies suggest that the rate and extent of tissue repair as a response to the injury inflicted by toxicants determines the ultimate outcome of hepatotoxicity (*3–21*). Blockage of the tissue repair leads to progression of injury, culminating in hepatic failure and death (*9–16*). Because stimulation of tissue repair is a biologic response that accompanies injury, quantifying this response in addition to measuring injury might be helpful in predictive toxicology.

Previous Studies

Earlier studies that form the basis of our present investigation indicated that tissue repair plays an important role in the progression of toxicity [see reviews by Mehendale (*1,2*)]. Prior exposure to a nontoxic level of chlordecone (10 ppm for 15 days) results in a marked amplification of carbon tetrachloride (CCl_4) hepatotoxicity and lethality. Neither the close structural analogs of chlordecone, mirex, and photomirex nor phenobarbital exhibit this propensity for increased lethality. Chlordecone also potentiates the hepatotoxicity and lethality of chloroform ($CHCl_3$) and bromotrichloromethane ($BrCCl_3$). Although the toxicity of these closely related halomethanes is potentiated by such low levels of chlordecone, the toxicity of structurally and mechanistically dissimilar compounds like trichloroethylene and bromobenzene is not potentiated. This remarkable capacity to potentiate halomethane hepatotoxicity is not related to chlordecone-induced cytochrome P450 or associated enzymes, enhanced bioactivation of CCl_4, increased lipid peroxidation, or decreased glutathione. These and other candidate mechanisms were considered carefully in experiments designed to verify their adequacy and were found inadequate; additional experiments revealed that tissue repair plays an important role in the progression of toxicity (*2*).

Further studies designed to investigate the underlying mechanisms demonstrated

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Abbreviations used: ALT, alanine aminotransferase; ATP, adenosine triphosphate; 2-BE, 2-butoxyethanol; $BrCCl_3$, bromotrichloromethane; CCl_4, carbon tetrachloride; $CHCl_3$, chloroform; *o*-DCB, *ortho*-dichlorobenzene; F344, Fischer 344; PCNA, proliferating cell nuclear antigen; S–D rats, Sprague–Dawley rats; SDH, sorbitol dehydrogenase; TGF-α, transforming growth factor alpha.

that ordinarily a low dose of halomethane such as CCl_4 is not lethal because of the stimulated cell division and tissue repair that occurs simultaneous to the infliction of liver injury (*17,18*). Subsequent studies revealed that the recovery from injury inflicted by a low dose of CCl_4, $CHCl_3$, or $BrCCl_3$ is due to the stimulation of cell division that occurs in two phases (*1,2,17–21*). First, a burst of cell division occurs as early as 6 hr after CCl_4 administration with a second larger wave of cell division occurring 36 to 48 hr after the administration of CCl_4. It is clear that the early burst of cell division is due to the mobilization of a small number of hepatocytes that are normally present in the liver in G_2 phase (*6,7*). During chlordecone + CCl_4 interactive toxicity abolishment in the early phase of cell division was observed. A number of proinflammatory cytokines (tumor necrosis factor alpha, interleukin-6), growth factors (epidermal growth factor, transforming growth factor alpha [TGF-α], hepatocyte growth factor), and protooncogenes (c-*myc*, c-*jun*, H-*ras*) are overexpressed during cell division, and these products of gene expression facilitate division of other cells in the tissue (*22–25*). If the early phase of cell division does not occur, the mechanisms and signals necessary to facilitate the neighboring cells would not occur, thereby preventing adequate second-phase cell division and resulting in progression of injury and animal death. By 24 to 36 hr, although the second phase of cell division does occur, in the face of unrestrained progression of injury this cell division and tissue repair comes too little and too late to restrain the accelerated progression of injury (*1,2,17,21*).

Cellular Bankruptcy in Adenosine Triphosphate Leads to Failure in Tissue Repair

In chlordecone + CCl_4 combination treatment, low levels of hepatic adenosine triphosphate (ATP), a consequence of precipitous glycogen depletion triggered by Ca^{2+} flooding, may be the reason for failure of hepatocytes to divide (*26–28*). Depletion of ATP appears to be due to the stress of cellular injury and Ca^{2+} extrusion through ATP-driven Ca^{2+} pumps. Studies designed to investigate the role of hepatic energy status using fructose 1,6-diphosphate or ATP as an externally supplemented source of energy have provided evidence consistent with the energy hypothesis (*29–31*). Further studies designed to test the validity of this concept using cyanidanol, a compound that increases hepatic ATP levels, revealed significant protection (*32,33*). A time-course study of the biochemical and histomorphometric analysis of cell proliferative activity indicates that the hepatoprotective action of cyanidanol is not due to mitigation of the early events leading to liver injury. The findings are more consistent with cyanidanol mobilizing the cellular biochemistry to stimulate the S-phase of the cell cycle through the availability of ATP. In the presence of hepatotoxic stimulus represented by hepatocellular necrosis, the cells in S-phase divide, providing new cells for hepatolobular restoration, tissue healing, and recovery from injury. From these studies it seems reasonable to assume that the decrease in normal physiologic concentrations of ATP in the hepatocytes critically affects tissue repair and the restoration of hepatolobular architecture. Studies with direct administration of ATP to rats undergoing chlordecone + CCl_4 toxicity also supported this concept. Injection of ATP to chlordecone-pretreated rats at –1, +1, 3, 5, 12, 24, and 36 hr of CCl_4 injection resulted in 100% survival (*31*). A significant protection was observed in markers of liver toxicity. Further studies were designed to investigate the possibility of decreased metabolism of CCl_4 during ATP protection. Regardless of ATP intervention, approximately 75% of the administered $^{14}CCl_4$ was expired as unmetabolized CCl_4 within 6 hr in rats treated with the chlordecone + CCl_4 combination (*31*). Interestingly, the *in vivo* metabolism of $^{14}CCl_4$, as evidenced by $^{14}CO_2$ expiration, was significantly increased in chlordecone + CCl_4-treated rats receiving ATP. An increase in CCl_4 metabolism instead of the anticipated decrease indicates that the ATP protection cannot be attributed to the diminished bioactivation of CCl_4 (*31*). These findings suggest that ATP administration during the early phase of injury restores normal liver function and tissue-healing mechanisms, permitting restoration of hepatolobular structure and function and animal survival.

In contrast to these observations, exposure to higher levels of phenobarbital but the same low level of CCl_4 results in almost twice as much liver injury but does not lead to increased animal mortality (*34,35*). Depletion of ATP does not occur in these livers (*26,27*). Therefore, the only consequence of this highly toxic liver injury is to postpone the early phase cell division until 24 hr but not abolish it. The second phase of cell division is greatly stimulated (*26*). In combination, this wave of highly stimulated cell division and tissue repair leads to systematic restoration of hepatolobular structure and function, followed by full animal recovery.

A Two-Stage Model of Toxicity

The previously described studies led to the proposal of a two-stage model of hepatotoxicity. An intriguing aspect of this work with toxicologic interactions and effects on tissue repair and injury is the emergence of a concept that permits the separation of the initial events responsible for infliction of injury from subsequent events that determine the final outcome of that injury (Figure 1). Hormetic mechanisms are activated upon exposure to toxicants. Although the mechanisms responsible for triggering mobilization of biochemical events leading to cellular proliferation after the administration of a toxicant are not fully understood, it is clear that early events involved in tissue repair are the critical determinants for the final outcome of toxicity. Studies described here (*1–21,36–40*) support the concept of a two-stage model of toxicity (Figure 1). Stage one, the inflictive stage, comprises mechanisms of initiation of injury; the second stage determines the progression/regression of injury. The latter

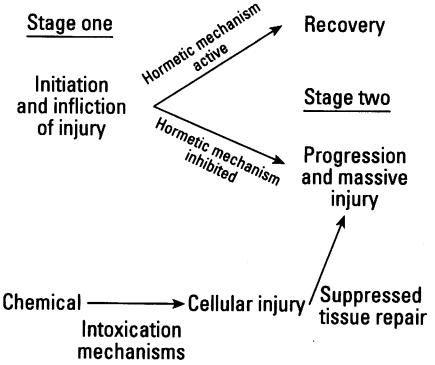

Figure 1. Scheme illustrating the proposed two-stage model of toxicity. Stage one involves infliction of cellular and/or tissue injury by intoxication mechanisms, which are well established for many chemical and physical agents. When injury is inflicted by a low dose of the offending agent (stage one), hormetic mechanisms such as cellular regeneration and tissue repair targeted for restoration of tissue structure are stimulated and complete recovery from injury follows with no additional consequence. If hormetic mechanisms are suppressed or ablated, the limited injury associated with exposure to a low dose of the offending toxic agent would continue unabated, resulting in progressive injury. High doses of toxic agents can cause ablation of stimulated tissue repair.

stage involves biologic response mechanisms of cell division and tissue repair initiated by a cascade of toxicodynamic events. These response mechanisms lead to restrained injury and full recovery from low to moderate doses of toxicants. It appears that irrespective of the mechanism of infliction of liver injury in the first stage, the ultimate outcome of hepatotoxicity, i.e., progression or regression of injury, depends on the timely and adequate appearance of tissue repair mechanisms.

Injury and Tissue Repair Are Opposing Toxicodynamic Forces in Predictive Toxicology of Chemicals

The characteristics of exposure to noxious agents and the spectrum of toxic effects come together in a correlative relationship, customarily referred to as the dose–response relationship, the most fundamental and pervasive concept of toxicology. This concept is used in predictive toxicology and consequently is a basic principle used in risk assessment. In developing dose–response relationships for toxic chemicals, at present only toxic injury is measured against a series of increasing doses. This information is incomplete and leads to erroneous predictions because it does not take into consideration the opposing and dynamic reparative and tissue restoration response. The above-described studies suggest that injury and tissue repair are simultaneous but opposing parallel responses to administration of toxic chemicals. If stimulated cell division and tissue repair are critical in predicting the ultimate outcome of toxic injury, then in addition to measuring injury in response to increasing doses of chemicals, it would be advantageous to measure the simultaneous but opposing response of stimulated tissue repair. This new dose–response paradigm would be more precise and accurate in predicting the final outcome of toxic injury.

Inclusion of tissue repair stimulation as the biologic event opposing injury may result in two sets of dose–response curves in the classic dose–response paradigm (Figure 2). At lower doses, as injury begins, a simultaneous but opposing tissue repair response appears, allowing the animals to overcome that injury. Predictably, these animals will suffer from injury but are rescued from progression of injury and death. As the dose increases, a threshold is reached where any additional increment in the dose will result in two adverse effects. First,

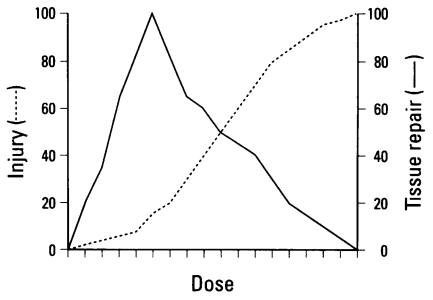

Figure 2. A typical dose–response relationship between the two opposing forces of inflicted injury and stimulated tissue repair upon exposure to a toxic chemical. As the dose increases, tissue repair is increased, allowing recovery from tissue injury. When the dose exceeds the threshold, tissue repair is attenuated and delayed, allowing injury to progress in an unrestrained manner and leading to organ or tissue failure and animal death. Quantifying both injury and adverse effect as well as stimulated tissue repair simultaneously as a dose–response relationship might be helpful in assessing the outcome of the interaction between these two opposing forces. The dose–response relationship can be used to explain interindividual differences, just as it can be used to explain differences among populations.

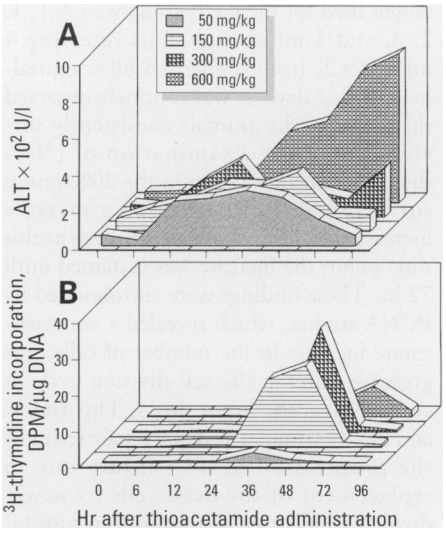

Figure 3. Dose response for liver injury and tissue repair after thioacetamide administration to rats. Male S–D rats were divided into four groups. At time zero, groups received ip injection of 50, 150, 300, and 600 mg/kg thioacetamide. Controls received normal saline. (*A*) Plasma ALT measured as a marker of liver injury. (*B*) [^{3}H]-thymidine incorporation into hepatocellular nuclear DNA measured as a marker of hepatocellular regeneration. Data adapted from Mangipudy et al. (*8*).

stimulation of tissue repair, which seems to be delayed with each incremental dose, is now much too delayed. Second, the amplitude of the tissue repair response is diminished. Therefore, decreased stimulation of tissue repair will result in unrestrained progression of injury and animal death. In addition to the use of the dose–response curve for prediction of the ultimate outcome in individual subjects, such a response can also be used for prediction in a population. To test this concept, we conducted studies with model hepatotoxicants (*8,41–43*).

Dose–Response Studies with Thioacetamide

The purpose of this study was to establish a dose–response relationship for thioacetamide, where tissue injury and repair were two simultaneous but opposing dynamic responses (*8*). Rats were injected with a 12-fold dose range of thioacetamide (50, 150, 300, and 600 mg/kg) and both liver injury and tissue repair were measured over a time course. Liver injury was assessed by serum enzyme elevations and by histopathology. Serum alanine aminotransferase (ALT) elevation did not show any dose response over a 12-fold dose range up to 24 hr (Figure 3A). A dramatic elevation in ALT was evident after 48 hr and only for the highest dose. Tissue regeneration response was measured by [^{3}H]-thymidine incorporation into hepatocellular DNA and by proliferating nuclear cell antigen (PCNA) procedure during a time course (6, 12, 24, 36, 48, 72, and 96 hr). Tissue regeneration, as indicated by [^{3}H]-thymidine incorporation in DNA, peaked at 36 hr after administration of a low dose of thioacetamide (50 mg/kg). With increasing doses, a greater but delayed stimulation of cell division was observed until a threshold (300 mg/kg) was reached. With a further increase (600 mg/kg) above the threshold dose, tissue repair was substantially delayed and diminished (Figure 3B), and injury as assessed by ALT elevations was remarkably accelerated, indicating unrestrained progression of injury leading to animal death. These findings suggest that, in addition to the magnitude of the tissue repair response, the time at which this occurs is critical in restraining the progression of injury, thereby determining the ultimate outcome of toxicity.

Dose–Response Studies with Carbon Tetrachloride

Similar studies were conducted with a 40-fold dose range of CCl_4 (*41*). The findings of this study were similar to those of thioacetamide described above. The dose

ranges used for the CCl_4 study were 0.1, 1, 2, 3, and 4 ml/kg (ip). Rats receiving 4 ml/kg CCl_4 (ip) experienced 80% mortality within 2 days. It was routinely observed that 20% of the animals consistently survived this dose. Examination of [^{3}H]-thymidine incorporation in the 20% group surviving at 48 hr revealed a massive increase (5-fold) in S-phase synthesis at this time point; the increase was sustained until 72 hr. These findings were corroborated by PCNA studies, which revealed a commensurate increase in the number of cells progressing through the cell division cycle as compared to the lower doses. This timely and highly stimulated tissue repair restrains the progression of liver injury due to replacement of the dead cells by newly divided resilient cells, resulting in restoration of the hepatic lobular structure in this group of surviving rats (*41*).

Dose–Response Studies with *o*-Dichlorobenzene

In these studies, male Fischer 344 (F344) rats were administered different doses of *o*-dichlorobenzene (*o*-DCB) (0.2, 0.6, 1.2 ml/kg, ip). Liver injury, as assessed by plasma ALT elevation and by histologic alterations, did not show a dose-dependent increase. The appearance of peak injury was delayed with an increased dose of *o*-DCB. With the lowest dose, injury peaked at 24 hr, whereas with the highest dose the injury peaked at 60 hr. Liver regeneration, as evaluated by [^{3}H]-thymidine and PCNA, showed dose-dependent increases at the lower two doses. Increasing the dose further to 1.2 ml/kg *o*-DCB resulted in delayed and diminished tissue repair response (*42*). These studies further support the concept that tissue repair is a function of dose and as the dose increases tissue repair increases only up to a threshold dose. Beyond the threshold, tissue repair is delayed, which allows liver injury to progress.

Dose–Response Studies with Trichloroethylene

In these studies rats were treated with a 10-fold dose range of trichloroethylene (250, 500, 1250, and 2500 mg/kg) and hepatotoxicity and tissue repair were studied over a time course of 0 to 96 hr (*43*). Light microscopic changes in hematoxylin-eosin-stained liver sections revealed a dose-dependent incidence of hepatic necrosis. However, liver injury as assessed by plasma sorbitol dehydrogenase (SDH) showed a dose–response over a 10-fold dose range only at 6 hr, whereas ALT did not show a dose response at any of the time points studied. Further studies designed to investigate the discrepancy between plasma enzymes and histopathology indicated that the lack of dose-related increase in SDH and ALT activities may be because of the inhibition of these enzymes by trichloroacetic acid, a metabolite of trichloroethylene. Tissue regeneration response as measured by [^{3}H]-thymidine incorporation was stimulated maximally at 24 hr after 500 mg/kg trichloroethylene administration. Higher doses of trichloroethylene led to a delay and diminishment in [^{3}H]-thymidine incorporation. These results further support the concept of dose-related increase in tissue repair up to a threshold dose and inhibition of tissue repair at doses above this threshold, leading to organ failure and animal death.

Dose–Response Relationships for Injury and Tissue Repair

A fascinating outcome of these dose–response studies is the dynamic relationship between the tissue repair response and the progression of the injury. Accelerated progression of liver injury becomes evident only after failure to elicit a prompt tissue repair response that culminates in liver failure and death. In all the previous examples tissue repair due to high dose is delayed and attenuated. Thus, a failure in timely and adequate appearance of tissue repair leads to an unrestrained progression of injury in high-dose treated groups. Approaches proposed in the literature to date for describing the dose–response relationship for cytotoxic chemicals such as chloroform have implicitly assumed that injury as measured by cell death or enzyme leakage is coupled in a one-to-one relationship with repair, as measured by cell division (*44–46*). However, the true functional relationship can be investigated by the application of statistical methods to test various hypotheses regarding the temporal and causative interactions between injury and repair. This dynamic interplay can be stated in a biologically based empirical mathematical model:

$$\text{Outcome} = f(\text{repair, injury})$$
$$\text{Outcome} = f\{\text{repair}(t) - \text{injury}(t)\}\,dt$$
$$\text{Outcome} = f\{\text{repair}(t) \times W_r(t) - \text{injury}(t) \times W_i(t)\}\,dt$$

where $W_r(t)$ is the weight given to repair (cell division), $W_i(t)$ is the weight given to injury (cell death). Based on our preliminary experiments, we suggest that for a given dose the ultimate outcome of injury is a function of the net difference between repair and injury and that this difference can be integrated. This model may provide greater precision in risk assessment because this model is biologically based and it takes into account the dynamic nature of tissue repair and tissue-healing processes. If experimentally validated, this concept might be more useful in improving the current paradigms of predictive toxicology and the science behind it.

Role of Stimulated Tissue Repair in Auto- and Heteroprotection Models

Stimulated cell division and tissue repair by exposure to toxicants has several implications. Clearly, the newly divided cells are available to restore tissue structure and function (*1,2*). A second aspect of this phenomenon appears to be the extra resiliency that comes with newly divided cells. Several investigators have reported that newly divided cells are resistant to the toxic action of a variety of chemicals (*47–55*). It appears that this mechanism may work to restrain the progression of injury in a tissue where cell division and tissue repair are permitted to occur. These concepts have been tested in auto- and heteroprotection models.

Autoprotection

If stimulated tissue repair is critical for animal survival upon administration of a toxic chemical at a lethal dose, one should be able to protect animals from the lethal action of an ordinarily lethal dose by stimulating tissue repair in advance by preexposure to a low dose of the same compound. Studies have revealed that CCl_4 autoprotection is due to the stimulation of cell division and tissue repair induced by preexposure to a low dose of CCl_4 administered 24 hr prior to the administration of the lethal dose (*10–13*). In a similar fashion, thioacetamide autoprotection is also due to the preplacement of tissue repair stimulated by a low dose of thioacetamide (*15*). Antimitotic intervention with the stimulation of tissue repair by colchicine leads to abolition of autoprotection in both the models (*9,11*). In the experiments with CCl_4, neither bioactivation nor the disposition of CCl_4 were affected (*11,12*). Exposure to phenobarbital postpones the early phase of cell division to 24 hr (*47*). If stimulated cell division is the primary mechanism responsible for CCl_4 autoprotection,

administration of the priming dose in phenobarbital-induced animals should lead to postponement of maximal autoprotection by approximately 24 hr. Autoprotection experiments with phenobarbital-induced animal models revealed that maximal autoprotection was delayed by 24 hr in comparison to autoprotection in naive animals (*47*). These experiments also revealed that destruction of cytochrome P450 by the priming dose of CCl_4 was not the primary mechanism of autoprotection. The discovery of stimulated tissue repair as the underlying mechanism of CCl_4 autoprotection has far-reaching implications for the toxicology of chemical combinations and chemical mixtures.

Heteroprotection

The experiments previously described suggest the possibility of protecting animals from a lethal dose of a compound by preplacing stimulated tissue repair in animals using any toxic chemical. Studies in which a low dose of thioacetamide was used to preplace stimulation of cell division have confirmed this concept, adding additional evidence in support of the key role of tissue repair in ultimate toxic outcome regardless of the extent of injury. Rats receiving a lethal dose of acetaminophen 36 hr after administration of thioacetamide (50 mg/kg) are fully protected from acetaminophen-induced lethality (*48*). Detailed studies revealed that neither the disposition of acetaminophen nor the bioactivation and infliction of acetaminophen liver injury are affected by prior treatment with thioacetamide. Preplaced stimulated tissue repair appears to sustain continued tissue repair, restrain acetaminophen-induced liver injury from progression, and enable the animals to recover from the normally lethal injury of acetaminophen (*48*).

Resistance of Newly Formed Cells to Toxicity

Because of the complex makeup of the liver, designing definitive experiments to test whether and how much the resiliency of newly divided cells contributes to recovery from toxic injury presents formidable difficulties. Therefore, this concept was tested in a simpler extrahepatic tissue model. To test the role of tissue repair and newly formed cells, studies were conducted with 2-butoxyethanol (2-BE), a glycol ether. These studies have led to greater insights in this regard (*50,51*). When given at toxic doses, 2-BE causes massive hemolysis, which leads to secondary toxic effects ultimately resulting in animal mortality. Prior repeated exposure to low levels of this chemical was reported to cause tolerance against 2-BE-induced hemolysis (*56*). Based on this concept an autoprotection model of 2-BE was established (*50,51*). Animals receiving one single moderately toxic dose of 2-BE experience an episode of hemolysis but are able to survive. During the following 7 days, animals replace all of the lost red blood cells through erythropoietic stimulation to restore their original hematocrit. At this point, when the animals are challenged with a lethal dose of 2-BE all the animals survive. The underlying mechanism was the resilience of newly formed red blood cells to hemolysis (Figure 4). The possibility that the survival afforded by prior exposure to a moderately toxic dose of 2-BE against a subsequent lethal challenge of the same compound could be due to the resilience of newly formed red blood cells was further tested. If newly divided cells are indeed the mechanism behind animal survival, protection should also be observed in animals that are bled and allowed to recover. Indeed, experiments in which the animals were bled to attain the same level of hematocrit obtained with a moderately toxic 2-BE-induced hemolytic episode confirmed that the mechanism behind this autoprotection was indeed due to newly formed red blood cells (Figure 4) (*51*). These findings have been pivotal in gaining new insights into biologic events that restrain injury.

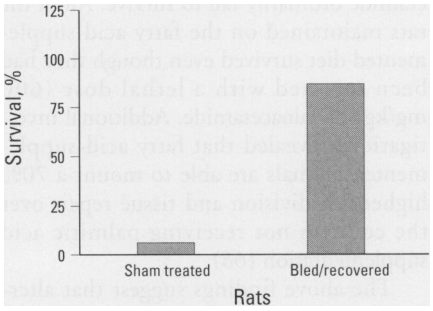

Figure 4. Resistance of newly formed red blood cells to 2-butoxyethanol hematotoxicity in animals; survival profile following lethal challenge with 2-butoxyethanol (1500 mg/kg). Rats were bled 2.5 ml/day for 3 days under light diethyl ether anesthesia. Blood-letting was followed by a 7-day period of recovery. Recovery was ensured by a return of depleted hematocrit values to prebled levels. Both bled and recovered rats as well as their sham-treated controls were challenged with a lethal dose of 2-BE and their survival was recorded. Data adapted from Cai and Mehendale (*49*).

Factors Affecting Tissue Repair

In addition to the dose of a chemical, several other factors affect the magnitude of tissue repair on toxicant exposure. Among these factors are some associated with the chemical characteristics of the toxicant. Others are associated with the host animal exposed to these toxic chemicals. Studies on species/strain differences, nutritional status, and age of the animal also emphasize the role played by these factors.

Species and Strain Differences in Tissue Repair

Species and strain differences in response to toxicant exposure are widely reported in the literature. Enormous differences in the extent to which tissue repair is stimulated have been observed between different species (*42,57–59*). For example, the 35-fold higher sensitivity of gerbils to CCl_4 in comparison to rats is due to sluggish tissue repair in gerbils (*58*). Substantial delay in the stimulation of tissue repair in gerbils results in unrestrained progression of even limited liver injury in this species. The bioactivation of CCl_4 in gerbils is approximately three times higher than in the rat (*57*), which translates into a higher infliction of injury in gerbils to begin with. However, increased infliction of liver injury in the gerbil as a result of higher bioactivation only partially explains the markedly higher sensitivity of this species to CCl_4 (*57–59*). Two lines of evidence are available to suggest that higher bioactivation only partially explains this high sensitivity. First, if gerbils are subjected to partial hepatectomy intended to stimulate tissue repair processes, bioactivation of subsequently administered CCl_4 is not diminished (*58*). However, survival of the partially hepatectomized gerbils from ordinarily lethal doses of CCl_4 suggests the critical role played by preplaced tissue repair on animal survival. Secondly, administration of drug-metabolizing enzyme inducers such as phenobarbital and mirex, which induce the isozyme responsible for bioactivation of CCl_4, results in an additional 2-fold increase in bioactivation (*59*). However, this does not lead to increased lethality in the gerbil. Although this has not been specifically investigated in gerbils, survival of the animals in spite of increased liver injury by prior exposure to phenobarbital may be related to increased tissue repair response, as was the case in rats (*47,59*).

F344 rats have been reported to be 75-fold more sensitive than Sprague–Dawley

rats (S–D rats) to liver injury of *o*-DCB (*60,61*). Whether this difference in liver injury is reflected in ultimate toxic outcome (survival/lethality) was not known. Studies revealed that the median lethal dose of *o*-DCB did not differ between F344 and S–D rats (*42*). In contrast to the reported 75-fold higher liver injury, we found only 10- to 15-fold higher injury in F344 rats compared to S–D rats (*60,61*). Lethality studies suggested that even though higher liver injury was evident in the F344 rats, this did not lead these animals to experience any higher mortality. Tissue repair stimulated in response to different doses of *o*-DCB (6-fold dose range) was compared between these two strains. These studies indicated that tissue repair response in the F344 rat is approximately 4 to 10 times higher than the S–D rat, suggesting that higher injury in F344 rat livers is of no consequence to animal survival because an exacting level of tissue repair stimulation rescues these animals (*42*). Similar to these studies, marked differences in CCl_4 toxicity among four strains of mice have been reported to be due to differential tissue repair (*62,63*).

These examples of species and strain differences also illustrate yet another important point. In animal-to-animal and animal-to-human extrapolation of toxicology data, uncertainty factors are often used because the mechanisms responsible for strain and species differences are not known. Risk assessors have relied largely on arbitrary uncertainty factors to take a more conservative and safer approach. It is becoming increasingly clear that a more scientific and rational approach might be to consider the two stages of toxicity in interstrain and interspecies extrapolation. We know relatively more about the differences in mechanisms responsible for inflicting injury among strains and species. Much less is known about the biologic toxicodynamic events that follow injury. In the absence of information regarding the mechanisms underlying species differences, we have often relied solely on the differences in bioactivation mechanisms as indicators of species differences. However, the previous two examples illustrate that the sole use of differences in bioactivation mechanisms as the basis for interspecies extrapolation cannot be justified.

Nutritional Status and Tissue Repair

Role of Glucose. Nutritional status is another host-related factor that significantly impacts tissue repair response. Carbohydrate substrates such as glucose are readily available sources of energy extensively used in emergency medicine. Some literature reports have suggested that glucose may inhibit stimulation of cell division and tissue repair (*64,65*). However, the impact of glucose as a source of energy on toxicant-induced injury and the ultimate outcome of that injury were not investigated. In recent studies the effect of glucose loading on thioacetamide hepatotoxic injury and lethality was examined (*37,66*). It was hypothesized that glucose loading would lead to higher lethality of thioacetamide. A hepatotoxic dose of thioacetamide (300 mg/kg) that is free from any toxicant-induced mortality was used in these studies. Although none of the glucose-loaded rats survived this dose of thioacetamide, all animals without glucose loading survived. Subsequent investigations revealed that glucose loading results in 70% inhibition of cell division stimulated by thioacetamide (*37,66*). In addition to thioacetamide, glucose loading also increased the mortality of CCl_4, $CHCl_3$, and acetaminophen, suggesting that this effect is not related to chemical structure or mechanism of infliction of injury (*37,67*).

Role of Palmitic Acid. In another set of experiments using thioacetamide as a model hepatotoxicant to inflict centrilobular injury, the diet of rats was supplemented with 8% palmitic acid (equicaloric with 15% glucose experiments) to investigate if supplemental fatty acids would enable these animals to survive a lethal dose of thioacetamide (*66–68*). Rats administered a 600-mg/kg dose of thioacetamide ordinarily fail to survive. All of the rats maintained on the fatty acid-supplemented diet survived even though they had been injected with a lethal dose (600 mg/kg) of thioacetamide. Additional investigations revealed that fatty acid-supplemented animals are able to mount a 70% higher cell division and tissue repair over the controls not receiving palmitic acid supplementation (*68*).

The above findings suggest that alterations in macronutrients such as glucose and fatty acids have a rather decisive and significant impact on the outcome of hepatotoxic injury. It should be noted that bioactivation mechanisms were not compromised in either of these two examples (*37,68*). Indeed, in the fatty acid-supplemented rats, bioactivation of thioacetamide was increased, which led to a corresponding level of increased liver injury (*68*). In spite of the increased injury, the animals were able to overcome this injury because of remarkably stimulated tissue repair processes. These findings suggest that nutritional differences in human diet are likely to contribute substantially to interindividual differences in toxicity (*66*).

Further studies were conducted to investigate the molecular mechanisms responsible for increased cell division after thioacetamide administration to rats fed the fatty acid-supplemented diet. Protooncogene expression plays an important role in stimulation of cellular proliferation during tissue regeneration. Liver regeneration is accompanied by a dramatic early increase in the expression of c-*myc* and other protooncogenes, which precedes the onset of increased DNA synthesis by at least 20 hr (*66,67*). Blockage of c-*myc* expression by indomethacin or decadron following partial hepatectomy impedes liver regeneration, suggesting the necessity of c-*myc* expression for the stimulation of DNA synthesis and cellular proliferation (*66*). A higher expression of c-*myc* was seen 48 hr after thioacetamide administration but not at 24 and 36 hr in the livers of rats fed a fatty acid-supplemented diet as compared to rats fed a normal diet. A higher c-*myc* expression at 48 hr in rats fed a fatty acid-supplemented diet is consistent with the observed higher S-phase synthesis rate in these rats at 72 hr as compared to rats on a normal diet. Higher expression of *p53* started at 6 hr in rats on a fatty acid-supplemented diet, but at 36 hr both the groups had similar expression. At 48 hr the expression of *p53* was lower in rats on a fatty acid diet as compared to rats fed a normal diet. Presumably, a higher expression of the *p53* tumor-suppressor gene helps inhibit cell division, which took place at 72 hr in the rats without fatty acid supplementation. Expression of the H-*ras* was same in both the groups, suggesting the probability of a minimal role played by this protooncogene in the protection provided by fatty acid supplementation against thioacetamide hepatotoxicity. Although definitive conclusions regarding the specific roles for expression of these protooncogenes await additional studies, these findings suggest that cause–effect relationships between protooncogene expression on the toxic outcome of hepatotoxicity are likely.

Role of Diet Restriction. The previously described studies suggest that xenobiotic-induced tissue repair is dependent on the source of cellular energy. Moderate diet restriction significantly

increases maximum and mean life span and prevents, delays, or retards the incidence of a plethora of age-associated diseases (*69*). More recently, the effect of moderate diet restriction on hepatotoxicity of thioacetamide has been investigated (*70–72*). Male rats were maintained on 65% of their *ad libitum* food consumption for a period of 3 weeks and then treated with a single low dose of thioacetamide (50 mg/kg). Maximal liver injury occurred in diet-restricted rats and was 6-fold greater than that observed in the group fed *ad libitum*. Histopathologic examination of the liver sections revealed liver injury concordant with plasma enzyme elevations. Interestingly, there was a higher and sustained S-phase stimulation in the diet-restricted rats as compared to the group fed *ad libitum*. PCNA studies revealed a corresponding stimulation of cell-cycle progression, indicating highly stimulated compensatory tissue repair. Although there was increase in injury, lethality experiments (600 mg/kg thioacetamide) indicated 70% survival in the diet-restricted group as compared to 10% survival in the *ad libitum* group. These findings suggest that although diet restriction increases hepatotoxic injury of thioacetamide, it protects from the lethal outcome by enhanced liver tissue repair. Because these findings raise the possibility that higher repair may be the result of higher injury, a study was undertaken with an equitoxic dose of thioacetamide.

Preliminary studies revealed that 600 mg/kg thioacetamide in rats fed *ad libitum* was equitoxic to 50 mg/kg thioacetamide in diet-restricted rats (*71,72*). At 12 and 36 hr the liver injury was almost equal in both the groups. A prompt and enhanced tissue repair response in diet-restricted rats at the low dose (6-fold higher liver injury) occurred, whereas at equitoxic dose (600 mg/kg), tissue repair in rats fed *ad libitum* was substantially diminished and delayed. The extent of liver injury was not closely related to the extent of stimulated tissue repair response. Light microscopy of liver sections revealed progression of hepatic injury in rats fed *ad libitum*, whereas by 120 hr injury regressed completely, leading to recovery in diet-restricted rats. Diet restriction resulted in abolition of the delay in tissue repair associated with the lethal dose of thioacetamide in rats fed *ad libitum*. This reversal of delay to restore sustained tissue repair response allows a significant number of diet-restricted rats to escape the lethal consequence (*71,72*).

Resiliency of Postnatally Developing Rats

Several studies have demonstrated that neonate and postnatally developing rats are resilient to a wide variety of structurally and mechanistically dissimilar hepatotoxicants such as galactosamine, acetaminophen, allyl alcohol, and CCl_4 (*49,73–78*). Most interestingly, young rats survive exposure to the lethal combination of chlordecone and CCl_4, which causes 100% lethality in adult male and female rats (*49,76,77*). In a study where postnatally developing (20- and 45-day), and adult (60-day) male rats were used, administration of CCl_4 (100 µl/kg) alone resulted in transient liver injury regardless of age, as indicated by plasma enzyme (ALT and SDH) elevations and histopathologic lesions. In chlordecone pretreated rats (10 ppm for 15 days), CCl_4-induced toxicity progressed with time, culminating in 25 and 100% lethality by 72 hr after CCl_4 in 45- and 60-day rats, respectively, in contrast to regression of injury without any mortality in 20-day rats. [^{3}H]-thymidine incorporation and PCNA studies revealed an association between delayed and diminished DNA synthesis, unrestrained progression of liver injury, and animal death. Time-course studies revealed that the loss of resiliency in the two higher age groups might be due to inability to repair injured liver rather than to infliction of higher injury.

Examination of growth factors and protooncogene expression revealed a 3- and 3.5-fold increase in TGF-α and H-*ras* mRNA expression, respectively, coinciding with maximal hepatocyte DNA synthesis in 20-day rats fed a normal diet, as opposed to only 2- and 2.5-fold increases observed in 60-day rats fed a normal diet, respectively (*77*). Increased expression of c-*fos* (10-fold) in 20-day rats occurred 1 hr after CCl_4 as compared to less than a 2-fold increase in 60-day rats. These findings suggest that prompt stimulation of tissue repair permits efficient recovery from injury during early postnatal development of rats.

Shifting Risk Assessment Paradigm for Public Health

During the last several decades substantial progress has been made in developing an understanding of the mechanisms by which chemical and physical agents initiate tissue injury (Figure 5). Once injury is initiated by these mechanisms of toxicity, cell death may occur if the cellular defense mechanisms are overwhelmed. If sufficient numbers of cells are destroyed, tissue function is compromised and animal death occurs. The ultimate toxic outcome lies within the outer bounds of recovery and death. Regardless of whether toxicity occurs from acute, subchronic, or chronic exposure, and whether it results in malignant or nonmalignant toxicity (all of which lie within the outer bounds of recovery and death as toxic outcomes), we in the public health sector are interested in increasing the possibility of recovery. Facilitation of recovery from injury and elimination of death are ideal goals in public health. Studies summarized here have revealed that distress signals (cytokines, growth factors, and other gene products) released during initiation of injury lead to stimulation of cell division to provide new cells to replace dead or dying cells. Stimulation of cell division is a normal dose-dependent endogenous response with a threshold that results in recovery from injury. Beyond the threshold, tissue repair is inhibited by delay in onset as well as attenuated response, which result in unrestrained progression of injury, organ failure, and death.

Increasing the tissue repair response and inhibiting the response yield substantial protection and enhancement of toxic outcomes, respectively. This basic principle is supported by a number of examples of

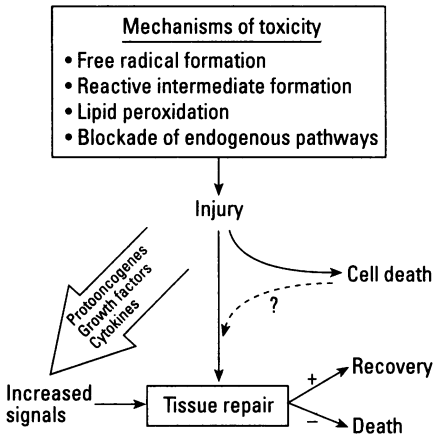

Figure 5. Mechanisms of toxicity. Once inside the body, chemicals cause injury by well-established mechanisms such as free radical formation, reactive intermediate formation, lipid peroxidation, blocking of endogenous pathways, etc. However, these mechanisms do not predict whether there will be recovery or if the injury will progress and lead to lethality. Progression or regression of injury and lethality depends on the timeliness and extent of tissue repair response. If tissue repair is active there will be recovery. If tissue repair is inhibited the injury progresses and the toxic outcome may be lethality.

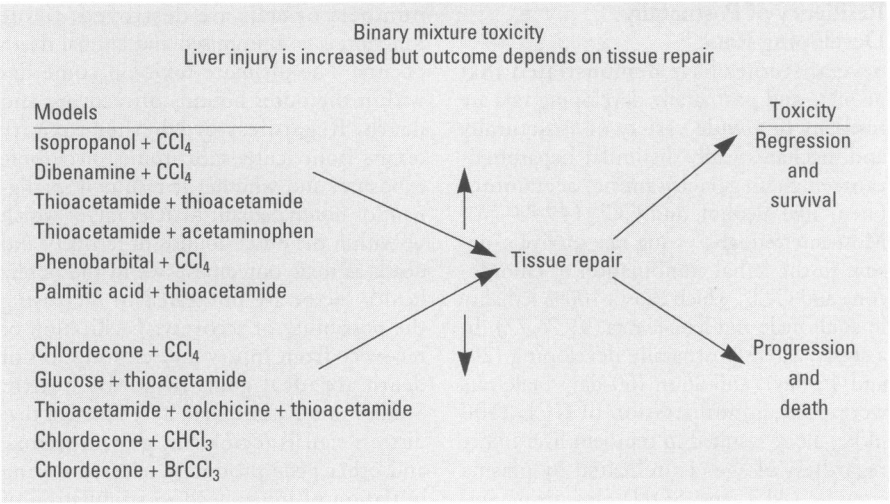

Figure 6. Binary mixture toxicity and the role of tissue repair. The combined action of two chemicals may potentiate or antagonize the injury and this potentiating or antagonizing action of the mixture depends on the stimulation or inhibition of cell division response. Stimulation of tissue repair speeds up recovery, whereas inhibition of tissue repair leads to progression of injury and animal death. Models are from references (*39,36,14,15,35,3,11,3,9,20,19*), respectively.

binary mixtures (Figure 6). Whenever tissue repair induced by one chemical is further enhanced by the binary combination of chemicals, the toxic outcome is survival. In contrast, if the tissue repair response is inhibited by the interactants in a binary mixture, the ultimate toxic outcome is predictably animal death (Figure 6). It should be noted that the extent of liver injury does not allow prediction of the ultimate outcome (*38–40,71,72*). For example, with the chlordecone + CCl_4 combination, although liver injury is less than 50% of that seen with phenobarbital + CCl_4 treatment (*38, 40*), rats receiving the chlordecone + CCl_4 combination die of hepatic failure, whereas those receiving the phenobarbital + CCl_4 treatment recover from hepatic injury and survive. Likewise, although isopropanol potentiation of CCl_4 toxicity results in higher liver injury, it does not lead to increased lethality (*39*), in contradiction to normal expectation based on liver injury. Furthermore, in diet-restricted rats, in spite of high liver injury, protection is observed (*71,72*). The reason for animal survival is greatly enhanced tissue repair (*71,72*).

Implication for Assessment of Risk to Public Health

Generally, mechanism-driven inflictions of toxicity or physiologic alterations are considered in risk assessment, but compensatory tissue repair response to injury has never been considered. Studies on compensatory tissue repair response will potentially have a marked impact on the way we conduct risk assessment, predictive toxicology, and public health. Significant advances in the understanding of mechanisms by which toxic chemicals inflict injury enable us to predict with a degree of confidence whether a given toxic chemical or physical agent will inflict tissue injury under a given set of exposure circumstances or not. However, the finding that the ultimate outcome of that injury is a result of the toxicodynamic and opposing interaction between two biologic responses of inflicted tissue injury and stimulated tissue repair suggests that a great deal more understanding of the underlying biology is essential before achieving greater precision in predictive toxicology and risk assessment.

Of immediate relevance in this regard are two important considerations. First, at least two levels of threshold doses can be suggested for toxic chemicals (Figure 1): One lower threshold dose at which cytoprotective mechanisms are overwhelmed and cell necrosis occurs and a second higher threshold dose above which the biologic compensatory response of cell division and tissue repair are compromised in two distinct ways—a significant latency in stimulating the tissue repair response and a significantly attenuated response. The inevitable combined effect of this compromise is the unabated progression of tissue injury, loss of organ function, and threat to survival.

Implications to Therapeutic Strategies

There is a universal acceptance of the concept that in stage one of toxicity, collectively all of the cytoprotective mechanisms offer a mechanistic basis for threshold dose above which cellular death will occur (Figure 1). Our studies reveal that in stage two of toxicity, there is also a tissue-based protective response (tissue repair) that increases with the dose until a threshold dose is reached (Figures 1 and 3). Between the two threshold doses there is a dose-related incremental biologic compensatory mechanism that effectively and promptly restrains tissue injury, permitting recovery from toxic injury. Stimulated cell division and tissue repair are the foundations of the biologic compensatory response (*2,8,41–43*). This suggests the possibility of therapeutic intervention in overcoming tissue injury regardless of the mechanism or the extent of initial infliction of that injury. Aside from impacting on safety and risk assessment based on molecular end points, these powerful concepts have potential as therapeutic and safety assessment tools in biomedicine and public health.

Modulation of Cell Division Due to Chemical Interactions May Lead to Decreased or Increased Threshold

Either of the two thresholds is subject to modulation as a result of interactive toxicity, particularly upon exposure to toxic chemical mixtures, resulting in decreased or increased infliction of injury during stage one. Examples of decreased or increased infliction of injury abound in the scientific literature. For example, the presence of drug-metabolizing enzyme inducers (or activators) or inhibitors can result in increased or decreased infliction of tissue injury, respectively, in stage one of toxicity. The resulting consequences of toxicity are well known and well described in the toxicologic literature.

However, the possibility of interactive interference by other chemical(s) at stage two of toxicity has not been examined extensively. Because toxicant-stimulated tissue repair response is critically involved in the ultimate outcome of toxicity, inhibition or enhancement of tissue repair by other chemicals may lead to unrestrained progression of injury and mortality or arrested progression of injury and recovery from injury and survival, respectively. Examples of both situations are available (*9–16*). In the highly amplified toxicity of CCl_4 by chlordecone, tissue repair is inhibited (*1,2*), which results in unrestrained progression of liver injury leading to 67-fold amplification of CCl_4 toxicity

by chlordecone. In contrast, although prior exposure to phenobarbital (*47*) or isopropanol (*39*) leads to highly potentiated infliction of liver injury, simultaneously enhanced tissue repair allows the animals to escape the lethal outcome (*39,47*). In diet restriction, highly augmented infliction of liver injury (6-fold higher) is of no consequence to animal survival because suitably augmented compensatory tissue repair is adequate to overcome liver injury (*70–72*). These findings suggest that even though massive (and ordinarily lethal) injury may occur because of modulation of stage one, enhanced tissue repair in stage two can compensate, thereby allowing the reversal of injury. Antimitotic intervention of cell division by colchicine administration after the mechanistic processes of infliction of injury (stage one) leads to progression of even limited injury culminating in lethal outcome from ordinarily nonlethal doses of hepatotoxicants (*9,11,79*).

In summary, the examples described here serve to illustrate the possibility that exposure to chemical mixtures may result in highly amplified lethal outcome, escape from anticipated increase in lethality, or significantly decreased injury. Currently, we are unable to predict whether and which chemical components of a chemical mixture may inhibit or enhance tissue repair and under what circumstances effects may occur. However, studies described here indicate the possibility that the threshold for lethal outcome may be lowered when one or more components of a chemical mixture inhibit cell division and tissue repair. Likewise, an increased threshold for lethal outcome is possible when one or more components of the chemical mixtures enhances the cell division and tissue repair. The third possibility is that no component in the chemical mixture may significantly modify tissue repair. Future effort should be directed toward identifying these possibilities using defined binary, ternary, and quaternary mixtures as model organic toxicants.

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